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Original Article

Thoughts modulate the expression of inflammatory genes and may improve the coronary blood flow in patients after a myocardial infarction

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ABSTRACT

Background: Mental stress is one of the main risk factors for cardiovascular disease. Meditation and music listening are two techniques that are able to counteract it through the activation of specific brain areas, eliciting the so-called Relaxing Response (RR). Epidemiological evidence reveals that the RR practice has a beneficial prognostic impact on patients after myocardial infarction. We aimed to study the possible molecular mechanisms of RR underlying these findings.
Methods: We enrolled 30 consecutive patients after myocardial infarction and 10 healthy controls. 10 patients were taught to meditate, 10 to appreciate music and 10 did not carry out any intervention and served as controls. After training, and after 60 days of RR practice, we studied the individual variations, before and after the relaxation sessions, of the vital signs, the electrocardiographic and echocardiographic parameters along with coronary flow reserve (CFR) and the carotid's intima media thickness (IMT). Neuro-endocrine-immune (NEI) messengers and the expression of inflammatory genes (p53, Nuclear factor Kappa B (NfKB), and toll like receptor 4 (TLR4)) in circulating peripheral blood mononuclear cells were also all observed.
Results: The RR results in a reduction of NEI molecules ($p < 0.05$) and oxidative stress ($p < 0.001$). The expression of the genes p53, NFkB and TLR4 is reduced after the RR and also at 60 days ($p < 0.001$). The CFR increases with the relaxation ($p < 0.001$) and the IMT regressed significantly ($p < 0.001$) after 6 months of RR practice.
Conclusions: The RR helps to advantageously modulate the expression of inflammatory genes through a cascade of NEI messengers improving, over time, microvascular function and the arteriosclerotic process.

1. Introduction

Mental stress is one of the major etiological aspects of ischemic heart disease and all its risk factors.[1–7] The persistent activation of the stress axis determines a state of low-grade chronic inflammation that feeds the atherosclerotic process and increases the risk of cardiovascular accidents.[1–3]

Meditating and listening to classical music, are two techniques that are able to turn off the brain areas that carry stress signals (the so called Default Mode Network) which evoke the Relaxation Response (RR)[8] through specific areas of the brain (called Attention Network).[2,3] The RR is aroused when an individual focuses on a word, a sound or a song, a phrase, a repetitive prayer, or a movement, disregarding everyday thoughts.[9] These two steps break the mind wandering and train of thoughts of everyday life. The practice of anti-stress methods (such as meditation or music appreciation)

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correlates with a significant decrease of adverse cardiac events in patients with myocardial ischemia, stroke, atherosclerosis, hypertension and heart failure[10] and are recommended by the American Heart Association (AHA). Please visit: http://www.heart.org/HEARTORG/HealthyLiving/Stressmanagement/StressManagment_UCM_001082_SubHomePage.jsp.

The numerous studies conducted so far in the field of meditation and music appreciation have associated to their practice a reduction in plasma concentration of hormones related to stress response (such as cortisol and norepinephrine), some inflammatory cytokines and oxidative stress mediators.[1–3] However, such research, focusing on individual neuro-endocrine-immune (NEI) mediators, do not offer an overview of how these biochemical messengers act in concert to determine the positive epidemiological effects related to the elicitation of the RR. Moreover, the final target of the NEI elements is cellular genome. We aimed to study some possible signals through which thought influences gene expression and many clinical, morphological, structural and functional cardiovascular parameters.

2. Methods

The design of our study follows what has already been published in literature[8] and is shown in Fig. 1. Our study consists of two elements: a prospective and a cross-sectional aspect. Each subject served as its own control. The cross-sectional aspect of the research involved the comparison between groups.

From October to December 2015, we enrolled 30 consecutive patients (23 males, mean age 55.9 ± 6.1) who were hospitalized in our Cardiology Wards for ST elevated (STEMI) or non ST elevated myocardial infarction (NSTEMI) and who suffered also from carotid atherosclerosis. All patients were free of cognitive deficit and had no other comorbidities. These patients were randomly divided as follows: 5 to Transcendental Meditation®, 5 to Pneumo-meditazione®, 10 to music appreciation. A brief description of each relaxation method is available in the online data supplements. Ten patients constituted the control group and were not subject to any intervention. They were asked to relax in a way that felt comfortable for them for a period of time corresponding to the relaxation session. Most of them chose to sit in their chairs with their eyes closed. Lastly, we enrolled 10 healthy control subjects matched for age and gender (5 of whom were trained in meditation and 5 in music appreciation). First of all these participants had to have a medical examination in order to verify their “good state of health”. None of the participants knew how to mediate or listen to music for relaxation reasons before the study. Each person signed a consent form giving their permission to participate in the study.

All patients were assessed by the Service of Clinical Psychology of our hospital in order to certify the individual personality characteristics, their neuro-cognitive reserve and the degree of perceived stress, through questionnaires used routinely and available in Internet (Mini Mental State (MMS), Esame Neuropsicologico Breve (ENB-2), Cognitive Reserve Index questionnaire (CRIQ), Symptom Checklist Questionnaire-Revised (SCL-90-R) and Perceived Stress Scale by Sheldon-Cohen). The same questionnaires were repeated after 6 months.

The initial four days of training took place in our hospital before discharge and the rest of the relaxation sessions were carried out independently by the subjects at home for 20 min, 2 times a day. After four days of training, we studied participants during the two daily relaxation sessions. At 8:00 a.m. vital signs were measured and blood samples taken at the start and immediately after the end of the session. In the afternoon, at 16:00 vital signs were measured again, and an electrocardiogram and transthoracic echocardiography (TTE) with assessment of coronary flow reserve (CFR) were performed before and immediately after the relaxation session. This same scheme was repeated after 60 days of daily practice at home. An echoDoppler of the supra-aortic trunks (SAT) was done pre discharge and after 6 months of regular daily practice to assess intima media thickness (IMT) in the carotid district. Thus, as the primary endpoint, we have audited the lowering of mean arterial pressure and the reduction of the genetic and biochemical parameters of inflammation (estimated effect size of at least 1.8 as described below). As a secondary endpoint, we evaluated the performance of other molecules that, conveying messages of stress in the body, affects the endothelial function of coronary microcirculation. Then, we performed an echocardiogram with CFR estimation and an echo-Doppler SAT to assess heart and microcirculatory function and the progress of atherosclerosis associated with the molecular mechanisms explored.

In blood samples we assessed the following NEI molecules: stress mediators (cortisol, corticotropin (ACTH), copeptin, epinephrine, norepinephrine, insulin, thyroid-stimulating hormone (TSH), growth hormone (GH), testosterone, dehydroepiandrosterone (DEHA-S), prolactin (PRL)), inflammatory markers (erythrocyte sedimentation rate (ESR), fibrinogen, highly sensitive C-reactive protein (HS-CRP), interleukin-6 (IL-6), transforming growth factor beta-1 (TGFβ-1), galectin-3), markers of oxidative stress (malondialdehyde (MDA), asymmetrical dimethyl arginine (ADMA)), a cellular stress marker (high mobility group box1

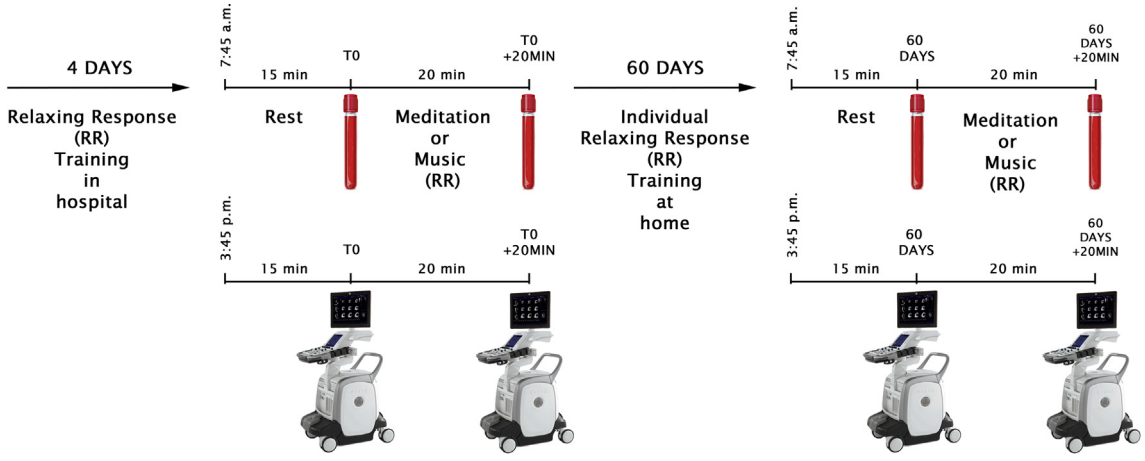

Fig. 1. Study design. Explanation in the text.

protein (HMGB-1)) and endocannabinoids (eCBs) (N-arachidonylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (DAG)) able to counteract the stress axis activation. The dosage of endothelial progenitor cells (EPCs) was also provided according to a well known procedure.[11] Moreover, we evaluated the expression of NFkB, p53 and Toll-like-receptor-4 (TLR4) genes in circulating peripheral blood mononuclear cells (PBMCs) in accordance with a procedure already described.[8] Lastly, we evaluated the lipid profile of the subjects (Total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides (respectively COL-T, LDL-COL, HDL-COL, Tg)). The environmental conditions at the time of data collection were the same for all the subjects. In particular, the training, the relaxation sessions and blood withdrawals have taken place in the classroom of our clinic situated alongside our echocardiography laboratory. Patient enrollment occurred on the seventh day after infarction and the four days of training were held in our classroom before discharge. All patients received optimal medical therapy in accordance with AHA and European Society of Cardiology guidelines for the treatment of ischemic heart disease and followed the same cardiac rehabilitation program (physical training and nutrition education). Medical therapy has been blindly adapted by colleagues of cardiac rehabilitation.

The variation of the parameters studied can be attributed to the practice of relaxation according to the methods used, because the precise timing of blood sampling (before and immediately after the end of the session) prevents any other influences. The daily practice carried out at home serves to promote and enhance the neurohormonal effect of relaxation, exactly in the same way as physical training helps to improve an athlete's performance during a competition. This means to be able, after 8 weeks, to confirm or increase the sensitivity to that observed at baseline. All groups were subject to the same environmental conditions, also the control patients were taken to our classroom for 20 min and did not undergo any intervention. They were simply asked to relax and most of them just sat down and closed their eyes.

Each investigator collected data of their own competence not knowing what kind of intervention the examined subject underwent. The only one to be aware of this was the first author who therefore did not take part in the blood samples collection and analysis, or in the measurement of vital signs and instrumental tests performed.

The study was approved by our institutional review board (Comitato Etico per la Sperimentazione Clinica-Azienda Sanitaria di Padova; protocol number 3487/AO/15).

3. Statistical analysis

The sample size was calculated based on the following scenario: type-I error rate of 0.05, power 0.95, pre-post changes in blood pressure and markers of inflammation, after intervention, from baseline, represented by an effect size of 1.8 (on the basis of the studies in the literature recently reviewed[1–3] and of a previous exploratory study performed in our clinic). Using a Wilcoxon signed-rank test (matched pairs), 5 sick subjects are required to detect this difference. Then, we analyzed the difference in the change of the parameters between patients' intervention groups and patients' control group and between patients' intervention group and the groups of healthy control subjects. In exploratory terms, a sample size of 5 persons allows to detect an effect size of 1.6 or higher with a type-I error of 0.05 and a power of 0.80, using a non-parametric test. Continuous variables are expressed as mean ± standard deviation or as median and interquartile range (if the variable does not have a normal distribution). Categorical variables are expressed as percentages and compared using the χ^2 test or Fisher's exact test. The comparison between the pre-post intervention changes was performed by means of Wilcoxon test. The comparison between groups was performed by means of the Mann–Whitney test. In the end, we compared the extent of the percentage changes of each parameter occurring during each relaxation session and after 8 weeks by means of the Mann–Whitney test. The distribution of the individual variables was assessed by the Shapiro–Wilk test. An initial comparison between groups was performed by means of Kruskal–Wallis test for independent samples or by Friedman test for paired data. Bivariate correlation was performed by Spearman test. Statistical significance was assumed if the null hypothesis could be rejected at $p = 0.05$. The statistical analysis was performed using software SPSS version 22.0 (Chicago, SPSS, Inc., Chicago, IL). All conditions that could have affected the improvement of the CFR and IMT have not changed and are comparable in the different groups at baseline, after 60 days and after 6 months (same physical rehabilitation and nutritional support, same therapy, same time of follow-up and same environmental conditions at the time of sampling).

4. Results

In our study we found no significant differences between relaxation techniques in terms of variance of each parameter before and after the sessions, both in the diseased population and in the healthy one. Therefore, we merged into a single “intervention” group all patients treated with meditation and music and into a single “intervention healthy controls” group all healthy subjects.

The study results are shown in Tables 1 and 2.

Patients (Table 1) are all Caucasians and live in the city of Padua. Patients' control group is made up of 80% males between 54.75 ± 7.93 years old while the “intervention group” 75% of males are aged 56.67 ± 12.01 years old. The healthy controls are males in 80% of the cases, aged 52.84 ± 15.2 years old. Patients tend to be overweight (BMI 26.2 ± 4.1) and 50 % of them suffer from dyslipidemia. Half of them had performed an exercise test (cycle-ergometer or myocardial scintigraphy) with negative results during the three months prior to the myocardial infarction. Coronary angiography performed on admission revealed the presence of pathology of two or three coronary vessels. 8 control patients and 16 patients in the “intervention group” reported a stressful event during the 6–12 months (7 ± 4.5 months) preceding the myocardial infarction (bereavement, divorce, business/economic problems). From the psychological point of view there were no cognitive deficits, and all patients had high levels of perceived stress with symptoms linked to it such as exhaustion, fatigue, insomnia, lack of concentration, lack of appetite, gastritis and bowel alterations, a condition called “sickness behavior”.[12,13] After 6 months, the significant improvement of the overall SCL90-R score ($p < 0.001$ in the intervention group and $p < 0.05$ in the intervention healthy control group) is to be referred to the notable improvement of the score in the subscales of “somatization”, “anxiety” and “hostility” with a trend towards significance for the parameters “depression” ($p = 0.059$) and “sleep” ($p = 0.055$). After 6 months, some patients have stopped taking beta blockers for excessive bradycardia or ACE inhibitors for hypotension.

Following the main results of the study presented in Table 2.

4.1. Vital signs and electrocardiogram

We observed significative changes in vital signs after the RR elicitation: the systolic blood pressure is lowered ($p < 0.0001$ after 60 days in both intervention groups), the heart rate ($p < 0.05$) and the respiratory rate ($p < 0.0001$) decreased and even the body temperature is lower ($p < 0.0001$) in both intervention groups.

Table 1
Characteristics of the population and results of psychological and clinical follow-up at 6 months.

	No intervention				Intervention				Intervention healthy controls	
Gender Male (%)	80				75				80	
Age (years)	54.75 ± 7.93				56.67 ± 12.01				52.84 ± 15.2	
Familiarity for CAD (%)	50				55				15●	
Smoke (%)	30		EX = 25		40		EX = 10		0●	0●
BMI (kg/m^2)	26.23 ± 3.91				24.48 ± 1.53				22.72 ± 2.85	
Hypertension (%)	0				0				0	
Diabetes (%)	0				0				0	
Dyslipidemia (%)	45				40				15●	
GERD-gastritis (%)	90				85				15●	
Allergy (%)	25				30				30	
Diarrhea/constipation (%)	70				75				40	
Negative stress test (%)	50				50				–	
NSTEMI (%)	50				60				–	
Number of coronary diseased (n = %)	1 = 30	2 = 20	3 = 50		1 = 27	2 = 23	3 = 50		–	–	–
Troponin (ug/L)	25.97 ± 13.05				32.07 ± 41.78				–	
Mediterranean diet (%)	100				100				100	
Hours exercise/Week	4.5 ± 0.58				4.14 ± 1.34				4.5 ± 1.04	
Sickness behavior (%)	100				100				30●	
Stressor events (%)	80				80				0●	
MMS (mini-mental state)	29 (28.75–29.25)				29 (27.75–29.25)				29.5 (28.25–30)	
ENB-2 (Esame neuropsico breve-2)	75.32 (69.33–81.89)				74,6 (72.09–79.98)				93.86 (91.11–97.24)	
CRI (Cognitive reserve index)	107,5 (94,5–119,25)				94,5 (89,25–99,25)				105 (100–107)	
SCL-90-R at discharge/after 6 months	0.34 (0.25–0.64)		0.35 (0.29–0.65)◆		0.48 (0.36–0.62)◆○		0.29 (0.21–0.45)◆❖○		0.23 (0.21–0.33)◆✚	0.09 (0.06–0.11)❖✚
Perceived stress scale at discharge/after 6 months	19.5 (19–20.25)		20 (19.5–21.25)❖		20 (18–21)◆○		12 (10–13)❖❖○		14.5 (11.75–18)◆✚	2.5 (2–3)❖✚
General health at discharge/after 6 months	65 (51.25–76.75)		62 (49–72)◆		72 (40–82)✚		75 (67–87)◆✚		81 (77.75–89.5)✚	87 (87–93)✚
Mental health at discharge/after 6 months	72 (63–78)		70 (64–76)◆		68 (52–80)✚		80 (76–84)◆◆✚		78 (73–80)✚	96 (90–99)◆✚
Vitality at discharge/after 6 months	50 (48.75–56.25)		50 (46.25–55)◆		60 (45–70)✚		70 (55–80)◆✚		62.5 (52.5–68.75)✚	77.5 (75–80)✚
Emotional role at discharge/after 6 months	50 (33.33–75)		50 (33.33–75)◆		66.67 (66–100)		100 (100–100)◆		66.67 (66.67–91.67)✚	100 (100–100)✚
mg of drug at discharge	50 (50–50)	2.5 (1.88–2.5)	2,5 (2.5–2.5)	60 (40–80)	50 (50–100)	1,88 (1.25–2.81)	2,5 (2.5–2.5)	80 (80–80)	–	–
mg of drug after 6 months	100 (100–100)	2,5 (2.5–3.75)	2,5 (2.5–3.13)	40 (40–50)	50 (31.25–50)	1,25 (0.31–1.25)	2,5 (1.25–2.5)	30 (20–40)	–	–
	Metoprolol	Bisoprolol	Ramipril	Atorvastatin	Metoprolol	Bisoprolol	Ramipril	Atorvastatin	–	–

The comparison between groups was performed by means of the χ^2 test, Mann–Whitney test and Wilcoxon test. In red is shown the comparison of independent samples related to "intervention" group and "intervention healthy controls" group.
● $p < 0.05$ χ^2 test.
❖ $p < 0.0001$ Mann–Whitney independent samples.
◆ $p < 0.05$ Mann–Whitney independent samples.
○ $p < 0.001$ Wilcoxon paired samples.
✚ $p < 0.05$ Wilcoxon paired samples.

According to electrocardiographic parameters we noticed a PR and QT intervals augmentation ($p < 0.05$).

4.2. Echocardiographic features

All the echocardiographic parameters explored vary to relaxation with the exception of the stroke volume index. In particular, there was a significatively important increase in both the 2D and 3D end-diastolic (EDV) and end-systolic volumes (ESV) ($p < 0.001$) along with a reduction of the ejection fraction (EF) ($p < 0.001$). The Suga index and the global longitudinal strain (GL-strain) were both reduced with the RR ($p < 0.001$) but GL-strain increased remarkably after 60 days compared to the control group ($p < 0.05$). The E wave deceleration time increased considerably in the intervention groups, especially after 60 days of RR training ($p < 0.001$).

4.3. Stress hormones

In both intervention groups we observed an important reduction of cortisol, ACTH, copeptin, epinephrine and norepinephrine ($p < 0.001$ in patients and $p < 0,05$ in healthy subjects) after relaxation. On the contrary, insulin increased ($p < 0.05$) with the RR (Fig. 2). TSH increased after 60 days of RR practice in the intervention group compared with the controls ($p < 0.05$) and decreased in healthy subjects ($p < 0.05$). After 60 days, the prolactin was reduced in both intervention groups ($p < 0.05$) while the GH, testosterone and DHEAS increased ($p < 0.05$) (Fig. 3).

The variation of TSH, GH, testosterone, DHEAS and prolactin was assessed only after 60 days because their trend had already been described during the RR.[9]

4.4. Inflammatory mediators

A notable change in the levels of cytokines and markers of inflammation occurs, particularly in patients' intervention group where ESR, fibrinogen, HS-CRP and IL-6 are reduced ($p < 0.05$). The same thing occurred in the case of TGFβ-1 and the galectin-3 ($p < 0.001$). Interestingly, TGFβ-1 increased significantly ($p < 0.05$) after 60 days of training in both intervention groups compared to the control group (Fig. 4).

4.5. Oxidative stress molecules

It is important to note that after 60 days of training the RR determines a significant reduction ($p < 0.001$) of the values of MDA both during meditation and/or music appreciation. The alteration of ADMA values are appreciable after 60 days of training especially due to the increase with the relaxation ($p < 0.05$ in the healthy controls group and p value of the pre-post percentage change in ADMA's levels, after intervention, from baseline (Δ%20 min T1) <0.05 between intervention group and control group) (Fig. 5).

4.6. Endocannabinoids

The eCBs are higher in the population recovering from myocardial infarction. The AEA increases with relaxation ($p < 0.001$) and decreases after 60 days ($p < 0.05$); in healthy subjects AEA increases after 60 days of training ($p < 0.05$). DAG decreases as a result of the RR ($p < 0.001$) and after 60 days ($p < 0.05$), (Fig. 5).

4.7. Cellular stress signal

HMGB-1 decreases after the RR ($p < 0.001$) and increases after 60 days of training ($p < 0.05$), (Fig. 5).

4.8. Endothelial progenitor cells

After the RR elicitation, it's possible to observe higher levels of circulating EPCs CD133+/CD34+ ($p < 0.001$) (Fig. 5) and their precursors CD34+ ($p < 0.001$) and CD133+ ($p < 0.001$), especially in the group of patients. There are no substantial variations of KDR+/CD34+, KDR+/CD133+ cells and KDR+/CD34+/CD133+ cells.

4.9. Gene expression

The RR causes an ($p < 0.001$) attenuation of inflammatory gene expression (p53, NFkB and TLR4) in the PBMCs both in the session period and after 60 days (p value Δ%20 min T1, T2 and 60 days <0.001 between intervention group and control group) (Fig. 6).

4.10. Clinical outcome

As a result, in the patients' group, endothelial function improved as expressed by the highly significant increase in CFR during the RR session ($p < 0.001$) and after 60 days ($p < 0.05$) and a reduction ($p < 0.05$) of IMT in the carotid vessels after six months (Fig. 7). The increase of CFR at 60 days correlates with the decrease of IMT at 6 months in the "intervention group" (rho = −0803, p = 0.003). No correlation was found in control patients.

The lipid profile as well varies depending upon activation of the stress axis showing a slight decrease in COL-T ($p < 0.001$), LDL-COL ($p < 0.001$) and Tg ($p < 0.05$) after RR elicitation and after six months ($p < 0.05$), (Fig. 8).

We want to highlight the significantly important difference between the changes during the relaxation session (expressed as Δ %) of most of the parameters examined between intervention group and control group, that indicate variations contradictory to the considered parameters.

5. Discussion

This study demonstrates that meditation and listening to particular music frequencies cause a modification of inflammatory genes expression, by means of a highly significant change (and below the current values of "normality") in circulating levels of NEI messengers resulting in a clinically favorable impact (improvement in endothelial function and the initial regression of carotid atherosclerosis) in patients with CAD.

5.1. Differences across groups

Concerning the control group of patients there were no evident changes only opposite variations of the parameters analyzed (please refer to Table 2, p value A vs B). As mentioned before all the subjects were given 15 min of rest before starting the experiment (Fig. 1). The behavior of the NEI messengers and of the clinical and instrumental parameters in control patients should be consistent with the persistence of the so-called "mind-wandering" thinking.[1–3,9,14] In fact, in both periods of observation, during the 20 min with eyes closed, control patients claimed that they found themselves thinking about work or family problems, and planning their day. We can only be either relaxed or stressed and never both simultaneously. The data collected in control patients would therefore seem to suggest the need for a precise strategy, like a mind-body technique such as listening to particular sounds or meditating, to effectively modulate some biochemical signs of stress related to the way of thinking, emotions and behaviors in every day life, at least in our Western society.

Table 2
Clinical, biochemical and instrumental results.

	No intervention				Intervention		
	A				B		
	T0	T0+20 min	60 days	60 days + 20 min	T0	T0+20 min	60 days
Vital signs							
Systolic blood pressure (mmHg)	118 ± 6.3	120 ± 6.1	120 ± 5.3	122 ± 5.2	124.5 ± 5.7✚⚭	113.6 ± 5.2✚◆	116 ± 6.4■⚭
Diastolic blood pressure (mmHg)	77 ± 2.6	72.5 ± 8.6	74.5 ± 5.3	74.8 ± 6.6	77.5 ± 4.6	70.9 ± 7.9	75.4 ± 8.7✚
Heart rate (bpm)	62.8 ± 8.7	68 ± 8.4	67 ± 10.4	67 ± 10.5	65.8 ± 13.9✚⚭	60.3 ± 9.3✚	63.3 ± 10.2✚⚭
Respiratory rate (breath per min)	15.25 ± 1	14.5 ± 1.3❖	14.5 ± 1.7	13.5 ± 1.3❖	16.2 ± 1.2■⚭◆	11.5 ± 0.9❖■◆	13.7 ± 0.8■⚭
Oxygen saturation (%)	98.5 ± 0.6	98.5 ± 0.6	98.5 ± 0.6	98.5 ± 0.6	98.4 ± 0.6	98.6 ± 0.5	98.4 ± 0.5
Body temperature (°C)	36.38 ± 0.2	36.4 ± 0.2◆	36.3 ± 0.1	36.4 ± 0.1❖	36.3 ± 0.2○	36.1 ± 0.2◆○	36.3 ± 0.1■
Electrocardiogram							
Heart rate (bpm)	68 ± 10.7	67 ± 8.7	62.5 ± 9.9	63 ± 10.6	65.3 ± 7.2✚⚭	59.1 ± 7.6✚	61.1 ± 7.9■⚭
PR (msec)	166.3 ± 33.3	166.5 ± 31.3	164.5 ± 36.5	164.5 ± 35.4	161.3 ± 25.2✚	166.5 ± 20.8✚	162.4 ± 25.9○
QT (msec)	388.6 ± 28.3	387.8 ± 28.7	390.5 ± 29.8	391.8 ± 30.2	392.9 ± 32.8○	411.5 ± 40.1○◆	384.9 ± 23.3○
QTc (msec)	410.3 ± 12.3	410.5 ± 11.8	396 ± 18.3	396.3 ± 18.1	409.7 ± 22.1	410.9 ± 23.5	385.3 ± 24
Echocardiogram							
2D-EDV (ml^3/m^2)	67.2 ± 10.2	67.6 ± 9.3	69.3 ± 9.7	68.8 ± 9.7	63.4 ± 16.6○⚭	68.7 ± 16.7○	59.8 ± 15.1○⚭
2D-ESV (ml^3/m^2)	30 ± 4.5	30.7 ± 4.7	28.1 ± 4.6	28.8 ± 3.7	25.6 ± 10.2○⚭	30.3 ± 9.6○	23.5 ± 6.6○⚭
2D-EF (%)	55.4 ± 0.7	54.6 ± 1.5	59.5 ± 1	58.3 ± 0.8◆	60.5 ± 5.9○⚭◆	56.4 ± 4.3○	60.9 ± 3.1○⚭
3D-EDV (ml^3/m^2)	72.1 ± 10.6	70.8 ± 10.6	72.9 ± 9.4	71.2 ± 9.3	68.4 ± 17.9○⚭	73.8 ± 17.6○	64.9 ± 17○⚭
3D-ESV (ml^3/m^2)	32.5 ± 5.3	31.3 ± 5	29.3 ± 4,2	29.1 ± 4.1	27.2 ± 11.6○⚭	33.1 ± 10.1○	25.8 ± 7.6○⚭
3D-EF (%)	54.9 ± 0.7	55.8 ± 0.8	59.8 ± 1.8	59.2 ± 1.8◆	59.9 ± 5.1○⚭◆	55.5 ± 4.1○	60.3 ± 3.5○⚭
Suga ($mmHg/ml^3$)	1.9 ± 0.5	1.9 ± 0.5	2.2 ± 0.4◆	2.2 ± 0.3	2.9 ± 1○⚭	2.1 ± 0.7○	2.9 ± 0.9◆○⚭
3D-Suga ($mmHg/ml^3$)	1.8 ± 0.5	1.9 ± 0.5	2.1 ± 0.3◆	2.1 ± 0.4	2.6 ± 0.9○⚭	1.9 ± 0.6○	2.6 ± 0.9◆○⚭
GL-Strain (%)	−18.4 ± 1.5	−18.6 ± 1.4	−19.3 ± 1.5◆	−19.4 ± 1.6	−19.4 ± 3.3○⚭	−17.7 ± 3.2○	−21.8 ± 2.2◆○⚭
Stroke volume index (ml/m^2/beat)	38.4 ± 5.5	38.2 ± 5.2	42.4 ± 5.3	41.1 ± 5.8	39.1 ± 8.2	39.5 ± 8.1	35.1 ± 13.2◆
E wave deceleration time (msec)	181.3 ± 38.9	190.5 ± 48.2	168.5 ± 15.9◆	170.5 ± 17.1❖	195.2 ± 34.3○◆	222.9 ± 43,4○◆	201 ± 30.1◆○
CFR	2.66 ± 0.53	2.39 ± 0.25	2.96 ± 0.4	2.89 ± 0.5❖	1.83 ± 0.7⚭○◆	2.96 ± 1.1○	2.51 ± 0.6⚭○◆
Stress hormones							
Cortisol (nmol/L)	295 (284.4–316)	306 (295–333.5)	400 (351–412.5)	415 (363.5–424)◆	358,5 (300,5–391)○	283,5 (247,5–357)○	401,5 (315–440)○
ACTH (ng/L)	15 (14–16.5)	18 (17.5–23)◆	20 (18.5–21.5)	21 (19.5–22.5)◆	18 (15–25)○	15 (12,5–17,5)◆○	19 (17–26)○
Copeptin (ADH) (pmol/L)	7 (6.95–7.9)	9.6 (9.3–10.9)	6.6 (6.4–7.6)	7.2 (7.1–8.2)◆	4,95 (2,6–8,1)○⚭	4,1 (2,25–6)○	3,75 (2,15–5,45)○⚭
Epinephrine (nmol/L)	0.22 (0.16–0.24)⚭	0.25 (0.18–0.37)	0.1⚭	0.1	0,21 (0,15–0,37)○⚭◆	0,10 (0,10–0,27)○	0,10 (0,10–0,11)✚⚭
Norepinephrine (nmol/L)	2.38 (1.97–3.03)	3.93 (3.42–3.96)★	2.31 (1.91–2.57)	2.44 (2.41–2.93)◆	3,22 (2,5–4,28)○⚭	2,5 (2,12–3,07)★○	2,48 (2,05–3,09)○⚭
Insulin (mU/L)	22.3 (17.1–22.9)	12.2 (12–13.8)	11.5 (10.2–11.5)	9.8 (9.2–10.2)◆	10.3 (6.6–12.7)✚	15.4 (8.3–22.4)✚◆	8.2 (5.7–13.1)✚
TSH (ug/dL)	1.29 (1.13–1.31)		1.31 (1.16–1.33)❖		1.23 (1.16–1.33)⚭❖		2.12 (1.85–2.29)❖⚭◆
Prolactin (ug/dL)	9.3 (8.59–10.05)		8.9 (8.41–9.70)◆		8.8 (7.39–9.52)⚭❖		7.2 (6.59–7.96)◆⚭❖
Testosterone (ng/mL)	3.67 (3.32–4.19)		3.9 (3.46–4.36)		3.68 (3.03–4.47)⚭◆		4.79 (4.17–5.56)⚭❖
DHEA-S (g/dL)	168 (156.5–183)		177 (166–189)★		160.5 (141–192)⚭❖		224 (208.5–270)★⚭❖
GH (ng/mL)	2.03 (1.94–2.707)		2 (1.93–2.01)◆		1.96 (1.79–2.17)⚭◆		2.12 (1.98–2.41)◆⚭★
Inflammatory markers							
ESR (mm/h)	17 (10.5–17.5)◆⚭	23 (14–26.5)	3 (2.5–3.5)★⚭	6 (4.5–6)	21.5 (14–31.5)◆○⚭❖	18.5 (12.5–29)○❖	8 (5.5–16)★✚⚭
Fibrinogen (g/L)	3.7 (3.4–4.1)	4.1 (3.65–4.35)	3.2 (3.15–3.2)	3.3 (3.25–3.35)	4.4 (3.6–4.9)○⚭❖	3.7 (3.3–4.4)○❖	3.1 (2.9–3.4)✚⚭★
HS-CRP (mg/L)	0.88 (0.79–3.23)	1.86 (1.45–4.30)	0.75 (0.69–0.95)	0.79 (0.72–0.99)	4.3 (0.78–10.13)✚⚭★	1.4 (0.88–4.66)✚◆	0.78 (0.36–1.24)✚⚭
IL-6 (ng/L)	<2 (<2–2.4)◆	3.8 (2.9–4.3)	<2 (<2–3.6)	<2 (<2–3.7)	2.2 (<2–4.85)◆✚⚭	<2 (<2–2.6)✚	<2⚭
TGF1-β (ug/L)	36.3 (35.4–38.7)★⚭	42.7 (39.5–43.9)★	26.6 (25.1–31.7)◆⚭	29.4 (29.3–343)	29.5 (27.7–34.5)★○⚭	24.95 (21.8–28.1)★○	37.9 (35.9–43.8)◆○⚭
Galectin-3 (ng/mL)	11.8 (11.1–14.4)⚭	13.6 (12.6–16.4)	13.4 (12.4–15.5)⚭	13.6 (12.9–17.6)	14.2 (12.9–14.9)○⚭◆	13.5 (12.2–14.4)○	13.3 (12–14.8)○⚭◆
Oxidative stress markers							
MDA (umol/L)	0.14 (0.14–0.15)	0.15 (0.14–0.16)★	0.14 (0.14–0.15)❖	0.17 (0.16–0.18)❖	0.14 (0.13–0.15)○⚭◆	0.12 (0.10–0.13)★○	0.11 (0.95–0.12)❖○⚭◆
ADMA (uM)	0.57 (0.56–0.66)	0.50 (0.46–0.53)	0.58 (0.575–0.58)◆	0.49 (0.48–0.53)	0.54 (0.5–0,6)	0.51 (0.48–0.62)	0.53 (0.49–0.61)◆
Cellular stress marker							
HMGB-1 (ng/mL)	1.10 (1.07–1.12)	1.22 (1.17–1.25)◆	1.11 (1.10–1.14)◆	1.17 (1.15–1.18)	1.13 (1.04–1.18)○⚭★	1.06 (1.03–1.13)◆○★	1.3 (1.14–1.45)◆○⚭
Endocannabinoids							
AEA (ng/mL)	0.68 (0.60–0.69)	0.68 (0.60–0.70)	0.62 (0.56–0.64)★	0.65 (0.61–0.67)★	0.55 (0.49–0.65)○⚭❖	0.65 (0.61–0.78)○❖	0.39 (0.33–0.45)★○⚭
DAG (ng/mL)	9.2 (8.5–10.2)	8.8 (8.25–9.85)	8.8 (8.2–9.65)◆	8.55 (8.18–9.08)◆	9.05 (7.5–10.2)○⚭❖	8.3 (6.6–9.4)○❖	6.95 (6.2–8)◆○⚭❖
EPCs							
CD34+(a.n./m.n.c. × 500000)	128 (114.5–235)	123 (119–167)	176 (133–189)	153 (126–157)	140.5 (100–188.5)○◆	161.5 (141–210)○	118 (92–208.5)○
CD133+(a.n./m.n.c. × 500000)	89 (84–153)	91 (84.5–132)	72 (66.5–122.5)	56 (33–85)	108 (90–152.5)○	152 (113–179)○	82 (62.5–160)○
KDR+ CD34+/34 (a.n.)	4 (4–4.5)	3 (2–7)	2 (1–4.5)	8 (4–11.5)	1.5 (1–3)◆	2 (0.5–3)	5 (0.5–6)
KDR+ CD133+/133 (a.n.)	7 (4–7)◆	1 (1–3)	0 (0–1)	2 (1–3)	5.5 (2–8)◆	4.5 (3.5–7)	2.5 (1.5–9.5)
CD133+ CD34+/CD34+ (a.n.)	99 (81.5–164.5)	89 (75.5–125)	122 (67.5–143)	31 (17.5–71.5)◆	79 (58.5–104.5)○⚭	103 (80–135.5)○◆	122.5 (71.5–166.5)○⚭
KDR+ CD34+ CD133+(a.n.)	3 (2–3.5)	0 (0–0.5)◆	0	2 (1–2)	1 (0.5–1.5)◆	1 (0.5–3)◆	0
Gene expression in PBMCs							
p53 (2dct)	0.00967 (0.00467–0.0829)	0.01257 (0.00631–0.09255)◆	0.010783 (0.00376–0.03654)❖	0.02141 (0.009535–0.1014)❖	0.00769 (0.00541–0.01063)○⚭	0.00505 (0.00356–0.00743)◆○◆	0.00317 (0.00234–0.00529)❖○⚭❖
NFkB (2dct)	0.00769 (0.00242–0.01407)◆⚭	0.01266 (0.00433–0.02157)❖	0.01589 (0.00521–0.03271)❖⚭	0.01968 (0.00704–0.04768)★	0.00426 (0.00322–0.00548)◆○⚭◆	0.00282 (0.00188–0.00409)❖○◆	0.00205 (0.00124–0.00303)❖○⚭❖
TLR4 (2dct)	0.002142 (0.00081–0.00433)⚭	0.004793 (0.00165–0.1686)❖	0.01564 (0.00123–0.03689)⚭	0.02126 (0.00199–0.07591)	0.00157 (0.00103–0.00192)○⚭◆	0.00098 (0.00079–0.00154)❖○	0.00066 (0.00047–0.00096)○⚭

The comparison between groups was performed by means of the Mann–Whitney test and Wilcoxon test. In red is shown the comparison of independent samples related to "intervention" group and "intervention healthy controls" group.
❖ $p < 0.0001$ Mann–Whitney independent samples.
★ $p < 0.001$ Mann–Whitney independent samples.
◆ $p < 0.05$ Mann–Whitney independent samples.
■ $p < 0.0001$ Wilcoxon paired samples.
$p < 0.001$ Wilcoxon paired samples.
✚ $p < 0.05$ Wilcoxon paired samples.
⚭ $p < 0.05$ Wilcoxon paired samples 60 days.
Abbreviations used in the table: CFR: coronary flow reserve; echoDoppler SAT: echoDoppler of the supra-aortic trunks; IMT: intima media thickness; ACTH: corticotropin; TSH: thyroid-stimulating hormone; GH: growth hormone; DEHA-S: dehydroepiandrosterone; PRL: prolactin; ESR: erythrocyte sedimentation rate; HS-CRP: highly sensitive C-reactive protein; IL-6: interleukin-6; TGFβ-1: transforming growth factor beta-1; MDA: malondialdehyde; ADMA: asymmetrical dimethyl arginine; HMGB-1: high mobility group box1 protein; AEA: anandamide; 2-AG: 2-arachidonoylglycerol; EPCs: endothelial progenitor cells; PBMCs: peripheral blood mononuclear cells; TLR4: Toll-like-receptor-4; COL-T: Total cholesterol; LDL-COL: LDL cholesterol; HDL-COL: HDL cholesterol; Tg: triglycerides.

Intervention	Intervention healthy controls				p Value			p Value		
B	C				A vs B			B vs C		
60 days + 20 min	T0	T0+20 min	60 days	60 days + 20 min	p Δ% 20 min T0	p Δ% 20 min T1	p Δ% 60 days	p Δ% 20 min T0	p Δ% 20 min T1	p Δ% 60 days
Vital signs										
106.9 ± 4.7	114.7 ± 4.7	104.7 ± 4.3	112.6 ± 6.1	94.9 ± 6	**<0.01**	**<0.01**	**<0.01**	0.97	**<0.0001**	**<0.05**
71.4 ± 8.4	72.7 ± 7.8	68.3 ± 4.3	70.9 ± 3.6	62.6 ± 3.9	0.798	0.442	0.127	0.6	**<0.05**	0.78
58.6 ± 11.4	61.2 ± 10.1	57 ± 9.7	63.7 ± 6.5	55.1 ± 6.6	**<0.01**	**<0.01**	0.327	0.52	0.15	0.18
9 ± 1.2	14,83 ± 0,98	10,33 ± 0,82	12,43 ± 2,3	7 ± 1,29	**<0.001**	**<0.001**	**<0.05**	0.68	0.11	0.55
98.8 ± 0.4	98.3 ± 0.5	98.8 ± 0.4	98.7 ± 0,5	98.7 ± 0,5	0.665	0.327	1	0.57	0.29	0.5
36.0 ± 0.1	36.1 ± 0.3	35.9 ± 0.3	36.2 ± 0.1	35.9 ± 0.2	**<0.05**	**<0.001**	0.574	0.21	1	0.5
Electrocardiogram										
54.8 ± 7.2	69 ± 8.7	61.3 ± 6.7	68 ± 9.8	56.3 ± 8.4	**<0.001**	**<0.001**	0.665	0.95	**<0.05**	0.91
165.4 ± 25.2	162.8 ± 22.8	166.8 ± 21.1	162.8 ± 33.1	168.3 ± 32	**<0.05**	**<0.05**	0.684	1	0.55	0.55
399.4 ± 25.9	376.8 ± 26.7	380.5 ± 21.5	374.8 ± 22.7	385 ± 29.8	**<0.05**	**<0.05**	0.953	0.44	0.39	0.75
387.1 ± 17.2	399.3 ± 30.7	378.7 ± 38.3	384.3 ± 26.4	377.5 ± 33.2	0.212	0.684	0.446	0.13	0.57	0.29
Echocardiogram										
66.2 ± 14.9	64.5 ± 6.6	69.7 ± 7.7	67.5 ± 5	75.9 ± 4.3	**<0.001**	**<0.001**	0.08	0.63	0.41	0.55
29.3 ± 7.1	22.4 ± 2.5	28.4 ± 4.1	24.2 ± 1.7	33.6 ± 1.8	**<0.001**	**<0.001**	0.469	0.58	**<0.05**	0.95
55.8 ± 2.6	65.1 ± 4.2	59.2 ± 3.7	64 ± 4.1	55.7 ± 1.6	**<0.05**	**<0.001**	**<0.05**	0.24	**<0.05**	0.78
71 ± 17.1	69.3 ± 6.3	73.4 ± 4.6	70.4 ± 6	76.8 ± 5.2	**<0.001**	**<0.001**	0.192	0.3	0.64	0.18
31.9 ± 8.1	24.2 ± 2.4	30.1 ± 3.3	25.7 ± 2.6	33.5 ± 1.7	**<0.001**	**<0.001**	0.127	0.37	0.36	0.55
55.1 ± 2.5	65 ± 3.9	59 ± 4.5	63.3 ± 4.1	56.2 ± 2.2	**<0.001**	**<0.001**	**<0.05**	0.21	0.15	0.26
2 ± 0.6	2.8 ± 0.4	1.95 ± 0.4	2.6 ± 0.3	1.8 ± 0.2	**<0.001**	**<0.001**	**<0.01**	0.056	0.22	0.31
1.9 ± 0.5	2.6 ± 0.4	1.8 ± 0.3	2.5 ± 0.4	1.8 ± 0.2	**<0.001**	**<0.001**	**<0.05**	0.14	0.8	0.84
−18.9 ± 2.3	−21.9 ± 3.9	−19.7 ± 3.7	−21.2 ± 1.6	−19 ± 1.1	**<0.001**	**<0.001**	0.574	0.14	0.32	**<0.05**
35.5 ± 12.9	43.6 ± 5.9	42.3 ± 4.8	44 ± 5.9	42.7 ± 3.4	0.736	0.277	**<0.05**	0.14	0.29	0.24
244 ± 48.i2	164.1 ± 9.9	187.3 ± 8.2	196.6 ± 57.8	278.9 ± 45.6	0.185	**<0.05**	0.645	0.49	**<0.05**	0.66
4.89 ± 1.3	2.54 ± 0.5	4.19 ± 0.9	2.86 ± 0.5	4.9 ± 1.5	**<0.001**	**<0.001**	0.49	0.96	0.4	0.35
Stress hormones										
281,5 (242–316)	389 (323–441)	332,5 (290–364)	374 (337–392)	277 (245–295)	**<0.001**	**<0.001**	0.127	0.68	0.97	0.13
15 (10,5–16,5)	19 (15–34)	13,5 (11–17)	23,5 (11–32)	15 (8–23)	**<0.001**	**<0.001**	0.645	0.21	0.78	0.67
3,5 (1,75–4,75)	4,45 (3,9–7,7)	4,05 (3,2–4,5)	4,75 (3,8–9,5)	4,3 (3,3–7,6)	**<0.001**	**<0.001**	0.158	0.97	0.26	0.09
0,1	0,12 (0,10–0,23)	0,10 (0,10–0,11)	0,10 (0,10–0,11)	0,10 (0,10–0,10)	**<0.001**	**<0.001**	0.192	0.16	0.74	**<0.05**
1,99 (1,77–2,39)	2,86 (2,34–3,25)	2,07 (1,98–2,86)	1,71 (1,56–1,79)	1,27 (0,83–1,66)	**<0.001**	**<0.001**	1	0.91	0.49	0.21
11.2 (6.7–18.5)	6.7 (4.1–10.2)	7.9 (5–10.7)	7.3 (3.9–9.4)	8.4 (5–10)	**<0.01**	**<0.01**	0.327	0.21	0.86	0.55
	2.36 (2.31–2.44)		2.24 (2.22–2.31)				**<0.001**			**<0.05**
	4.69 (3.82–5.46)		3.80 (2.98–4.21)				**<0.001**			0.55
	9.75 (7.6–12.1)		11.25 (8.4–11.7)				**<0.001**			**<0.001**
	351 (323–389)		392,5 (375–426)				**<0.001**			**<0.0001**
	3.38 (3.21–4.87)		4.07 (3.48–4.99)				**<0.001**			0.49
Inflammatory markers										
5.5 (3.5–9)	4.5 (2–6)	3.5 (2–6)	4.5 (2–8)	3 (2–6)	**<0.001**	**<0.001**	0.505	0.57	0.8	**<0.05**
2.8 (2.6–3.1)	2.8 (2.4–3.1)	2.45 (2.3–3)	2.5 (2.3–2.6)	2.35 (2.2–2.5)	**<0.001**	**<0.001**	0.277	0.52	0.49	0.11
0.76 (0.34–1.2)	0.73 (0.39–1.36)	0.36 (0.16–0.88)	0.35 (0.16–0.69)	0.26 (0.16–0.47)	**<0.001**	**<0.001**	0.127	0.68	0.36	0.11
<2	<2	<2	<2	<2	**<0.05**	0.327	**<0.05**	0.07	0.64	0.09
31 (26.4–38.2)	32.5 (26.5–39.8)	28.5 (21.7–36.7)	46.2 (43.6–51.9)	39 (32–44.9)	**<0.001**	**<0.001**	**<0.001**	0.47	0.74	0.9
12.5 (11.4–14.4)	12.5 (12.1–13.3)	12.1 (7.8–12.5)	11.2 (8.2–11.9)	10.1 (8.1–10.1)	**<0.001**	**<0.001**	**<0.01**	1	0.17	0.31
Oxidative stress markers										
0.85 (0.75–0.1)	0.13 (0.12–0.13)	0.11 (0.10–0.11)	0.09 (0.09–0.10)	0.075 (0.07–0.08)	**<0.001**	**<0.001**	**<0.001**	0.97	0.64	0.36
0.55 (0.48–0.63)	0.49 (0.45–0.49)	0.49 (0.44–0.52)	0.50 (0.45–0.55)	0.54 (0.49–0.61)	0.1	**<0.05**	1	0.79	0.86	0.31
Cellular stress marker										
1.13 (1.08–1.22)	1.27 (1.25–1.31)	1.23 (1.16–1.25)	1.34 (1.3–1.59)	1.25 (1.23–1.29)	**<0.001**	**<0.001**	**<0.05**	0.27	0.91	0.6
0.48 (0.39–0.52)	0.24 (0.18–0.34)	0.38 (0.33–0.44)	0.33 (0.26–0.38)	0.51 (0.49–0.55)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.0001**
6 (5.35–7.28)	5.35 (4.40–6.20)	4.30 (3.20–5.20)	4.3 (3.5–5.1)	3.8 (2.8–4.05)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.05**
EPCs										
141.5 (110–219)	271 (119–319)	314.5 (160–372)	249 (116–276)	290 (124–354)	**<0.05**	**<0.05**	0.382	0.62	0.59	0.49
121.5 (84–170)	186 (78–243)	209 (158–244)	146 (64–170)	220 (68–248)	**<0.001**	**<0.001**	0.158	0.47	0.36	0.72
2.5 (0.5–6)	9 (4–17)	4 (1–7)	1,5 (0–4)	1,5 (1–2)	0.871	0.287	0.953	0.9	0.31	0.39
3.5 (1–5)	6.5 (0–10)	4 (3–13)	1.5 (1–2)	1 (0–2)	0.871	0.615	**<0.05**	0.35	0.75	0.32
160 (114.5–189)	138 (47–170)	176.5 (97–215)	191.5 (61–213)	217 (106–226)	**<0.001**	**<0.001**	**<0.01**	1	0.26	0.49
1.5 (0–2)	5 (0–10)	2,5 (0–6)	0 (0–0)	0.5 (0–1)	0.469	0.857	0.811	0.95	0.29	0.73
Gene expression in PBMCs										
0.00255	0.0045	0.00305	0.00129	0.00094	**<0.001**	**<0.001**	0.277	0.91	0.36	0.08
(0.00213–0.00416)	(0.00403–0.00594)	(0.00258–0.0046)	(0.00105–0.0017)	(0.00072–0.00132)						
0.00125	0.00253	0.00228	0.00056	0.0003	**<0.001**	**<0.001**	**<0.001**	0.27	0.09	**<0.05**
(0.00076–0.0021)	(0.00242–0.00274)	(0.00204–0.00258)	(0.00041–0.0007)	(0.00017–0.00051)						
0.00029	0.00112	0.00084	0.00045	0.00022	**<0.001**	**<0.001**	**<0.001**	0.91	0.36	0.72
(0.0002–0.00048)	(0.00091–0.00126)	(0.00059–0.00104)	(0.00028–0.00079)	(0.00018–0.0005)						

5.2. Effects of the RR

The results presented in Table 2 offer some of the mechanisms linked to the RR elicitation through musical frequencies or, on a psychic level, by bringing thoughts from a state of ordinary "mind wandering" to the so-called "meditative state."[1–3,9]

The RR results in a reduction of the body's metabolism as revealed in our study by the change of vital signs and of the body temperature specifically, with relative improvement in cardiac performance and a cardiac workload optimization. Echocardiographic variations are not "operator dependent" nor linked to a interobserver variability. Indeed, to overcome this methodological limitation, we took three-dimensional measurements (which confirm the 2D data) performed automatically by the echocardiographic machine software independently from the operator.[15] Therefore the echocardiographic changes presented are to be

Table 2 (*Continued*)
Clinical, biochemical and instrumental results.

	No intervention				Intervention		
	A				B		
	T0	60 days	60 days + 20 min	6 months	T0	60 days	60 days + 20 min
Lipid profile							
COL-T (mg/dL)	126 (123.5–159)	103 (101.5–134)	102 (101.5–133)	124 (119–138)	173.5 (161–213)*	124 (111–146)○◆	119 (107,5–140)○◆
LDL-COL (mg/dL)	79 (73.5–108)	67 (61–72)	66 (61–72)	72 (71–74)	117 (99.5–137.5)*⁘	73,5 (70.5–79.5)○	68,5 (66.5–75)○
HDL-COL (mg/dL)	45 (36–45.5)	46 (38–47)	45 (38–46)	46.5 (37–47)◆	45 (38–51)*◆	45 (41.5–53)✚◆	48 (43.5–54)✚◆
TG (mg/dL)	52 (47–92)	53 (49.5–93)	55 (49.5–93)	60 (56–94.5)	92.5 (75.5–118.5)	93 (76–124)○	89.5 (72–120.5)○
Echo-Doppler SAT							
I.M.T. (mm)	1.53 ± 0.31			1.63 ± 0.36	1.53 ± 0.3○		

related to the hemodynamic modifications associated with relaxation. In particular, the afterload and heart rate are reduced while there was no notable change in stroke volume. This, for the Frank–Starling mechanism, determines an increase of both the diastolic and systolic volumes and a decrease of myocardial contractility proportional to load conditions (described, in our work, by the reduction of the EF and Suga index). However, also the intrinsic cardiac contractility decreases as evidenced by GLS-strain variation associated with relaxation. These data indicate the ability of the myocardium to maintain an effective cardiac output with a lower oxygen consumption. This is especially important for a heart that has suffered an ischemic insult because, over time, the RR can increase its intrinsic myocardial contractility more significantly than the only pharmacological and rehabilitative interventions (significant GLS increasing at 60 days in the intervention group). In healthy subjects we have not documented any GLS significant changes at 60 days, probably because their basal myocardial contractility was already optimal having not suffered any myocardial insult.

The increase in diastolic time (increased PR interval on electrocardiogram and E wave deceleration time on echocardiogram) and the modification of hemodynamic parameters favor the coronary endothelial function and hyperemic flow (CFR). In fact, the CFR improves both because of a decrease in basal coronary flow, and an increase of the flow during adenosine infusion. The reduction in body metabolism and myocardial oxygen consumption (drop in body temperature, bradycardia, decrease in afterload) explain the reduction of the basal coronary flow.[16,17] The NEI changes may explain the increased vasodilatory reserve and consequently the RR. The improvement of endothelial function (which also resulted in the slight reduction of IMT in the carotid district) is not only due to mechanical/hemodynamic reasons. Indeed, following the RR, an attenuation of alarm signals that regulate endothelial function[18] occurs with the decreasing in circulating levels of the neuroendocrine mediators of stress (for example noradrenaline and cortisol) and inflammation. It has been described that mental stress induced myocardial ischemia occurs mostly in coronary subjects, is often asymptomatic and is associated with a worse prognosis and a

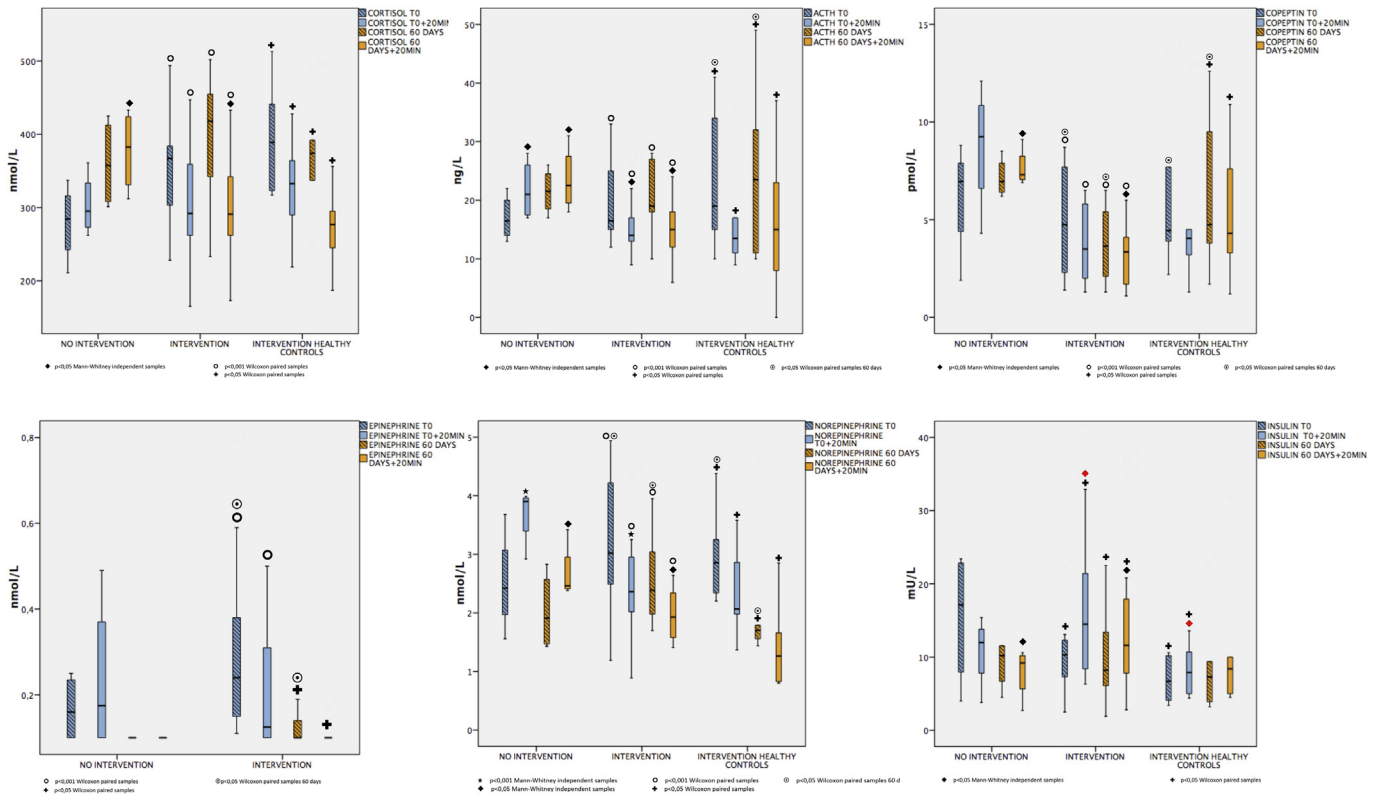

Fig. 2. Cortisol, ACTH, copeptin, epinephrine, norepinephrine and insulin variation during the RR elicitation session at the time of the first observation (blue) and after 60 days (orange). Abbreviations as in the text.

Intervention	Intervention healthy controls				p Vvalue			p Value		
B	C				A vs B			B vs C		
6 months	T0	60 days	60 days + 20 min	6 months	p Δ% 20 min T0	p Δ% 20 min T1	p Δ% 6 months	p Δ% 20 min T0	p Δ% 20 min T1	p Δ% 6 months
Lipid profile										
128 (121–139)⊛❖	177 (174–180)⊛	164 (161–168)◆○	159.5 (156–162)◆○	166.5 (163–170)❖⊛		**<0.01**	**<0.01**		0.09	**<0.05**
75,5 (74–77)⊛	79.5 (77–82)❖⊛	78.5 (75–80)○	75.5 (71–77)○	77.5 (76–78)⊛		**<0.001**	0.327		**<0.05**	**<0.05**
51,5 (48.5–57)◆⊛	53 (48–56)◆⊛	56.5 (54–60)◆✚	58 (56–62)◆✚	58 (55–62)⊛		**<0.05**	0.192		0.84	0.44
102.5 (85–120)◆	85 (79–91)⊛	84 (76–92)○	80.5 (74–89)○	84.5 (74–89)◆⊛		**<0.01**	0.721		0.97	0.09
Echo-Doppler SAT										
1.46 ± 0.28○							**<0.05**			

more aggressive atherosclerotic process.[19] Moreover, in healthy controls, both mental and exercise/dipyridamole stress tests induce increased myocardial blood flow in normal coronary vessels, as a result of coronary microvascular dilation. In patients with CAD, coronary flow during mental stress is lower in regions without epicardial stenosis than in those with significant stenosis, suggesting microvascular constriction.[20] The performance of the CFR that we have documented is consistent with these considerations and reflects an improvement in endothelial function due to mental relaxation.

The TGFβ-1 is a pleiotropic cytokine with anti-inflammatory and reparative/regenerative action.[21] Its levels decline as a result of the relaxation in line with the attenuation of inflammation reflected in the performance of the other parameters analyzed. Over time, there was an increase in baseline TGFβ-1. This phenomenon could represent the acquisition by the body of a greater ability to fight stress situations linked to the progressive downregulation of inflammatory genes found in the PBMCs. This behavior is also consistent with the results of the literature[22] relating to the functioning of the stress axis that, as a result of the RR training, optimizes its activity becoming much quicker to take action (greater slope of the curve cortisol after stress) and taking less time to return to homeostasis.[23]

There are many studies that have shown the correlation between mental stress and oxidative stress.[4,24,25] In our work we described the opposite side of the coin. In fact, the RR results in a reduction of body's oxidative stress as evidenced by the behavior of the MDA. ADMA increases during relaxation probably trying to down regulate the production of nitric oxide (NO) responsible for the vasodilatory response observed with the CFR data.

Endocannabinoids (eCBs) are molecules that counteract the stress reaction[26–30] and are synthesized from membrane phospholipids in the heart and other cardiovascular tissues. They activate several epigenetic mechanisms through cannabinoid receptors CB1 and CB2, transient receptor potential V1, peroxisome proliferator-activated receptors, and a vascular G-protein coupled

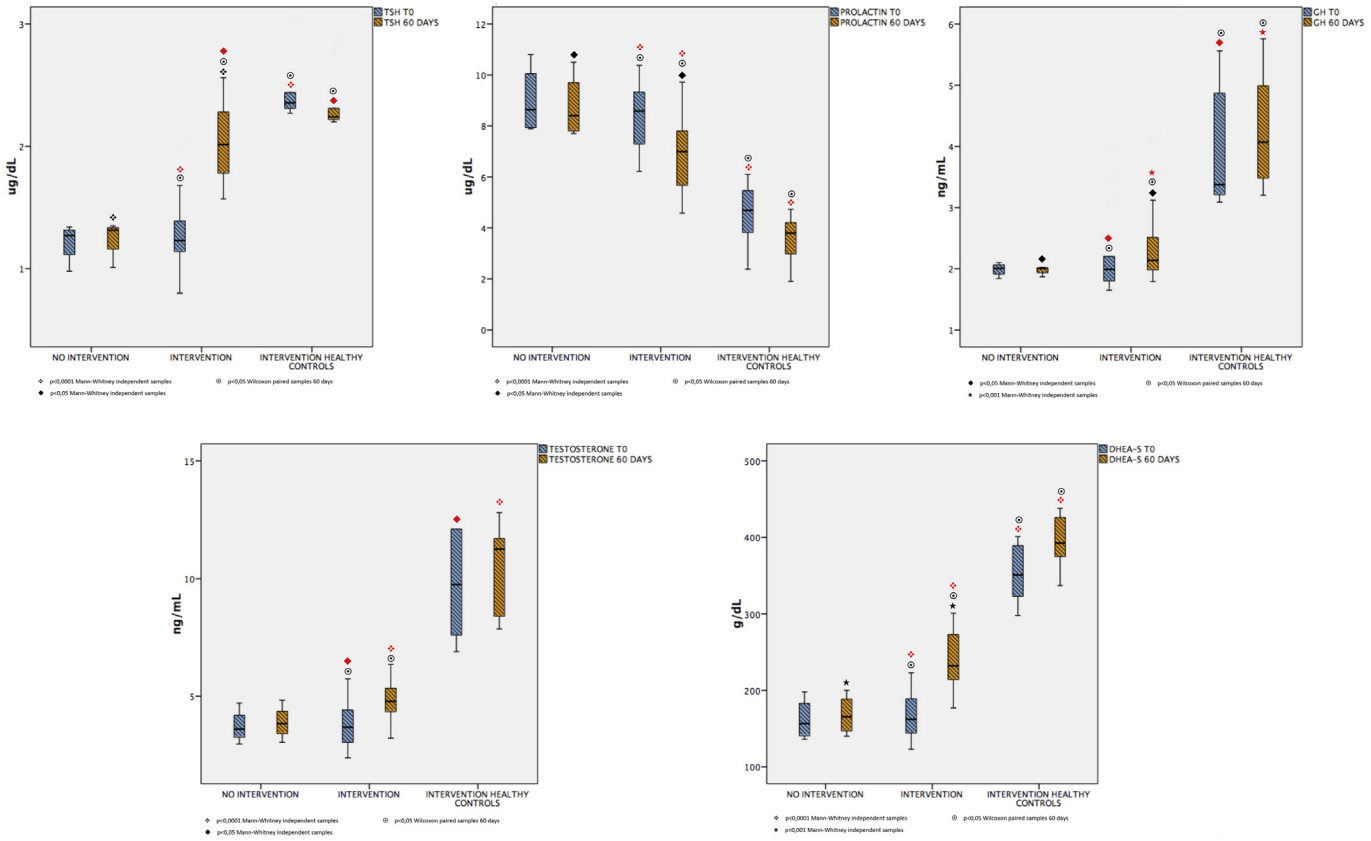

Fig. 3. TSH, prolactin, GH, testosterone and DHEA-S variation between the first observation (blue) and after 60 days (orange). The variation of these hormones was only assessed after 60 days because it has already been described their trend during the RR.[9] Abbreviations as in the text.

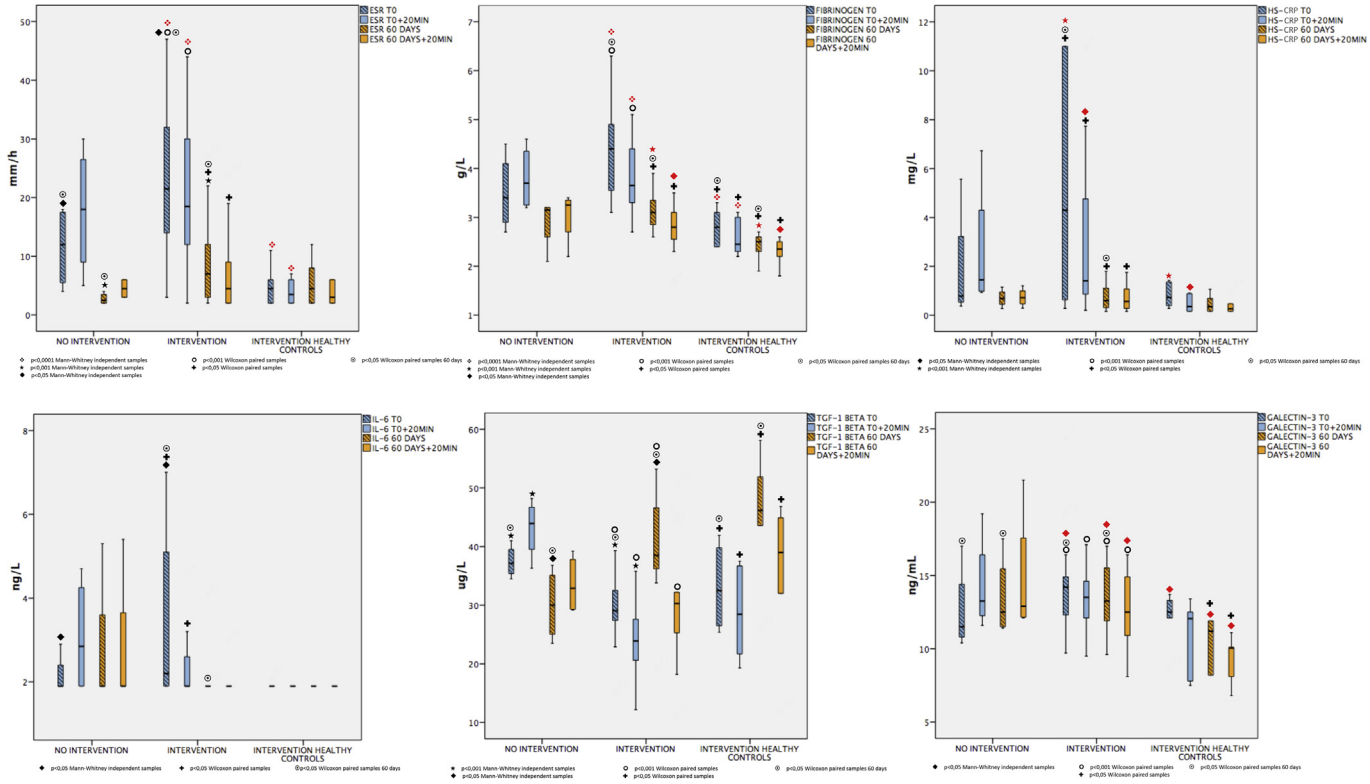

Fig. 4. ESR, fibrinogen, HS-CRP, IL-6, TGFβ-1 and galectin-3 variation during the RR elicitation session at the time of the first observation (blue) and after 60 days (orange). Abbreviations as in the text.

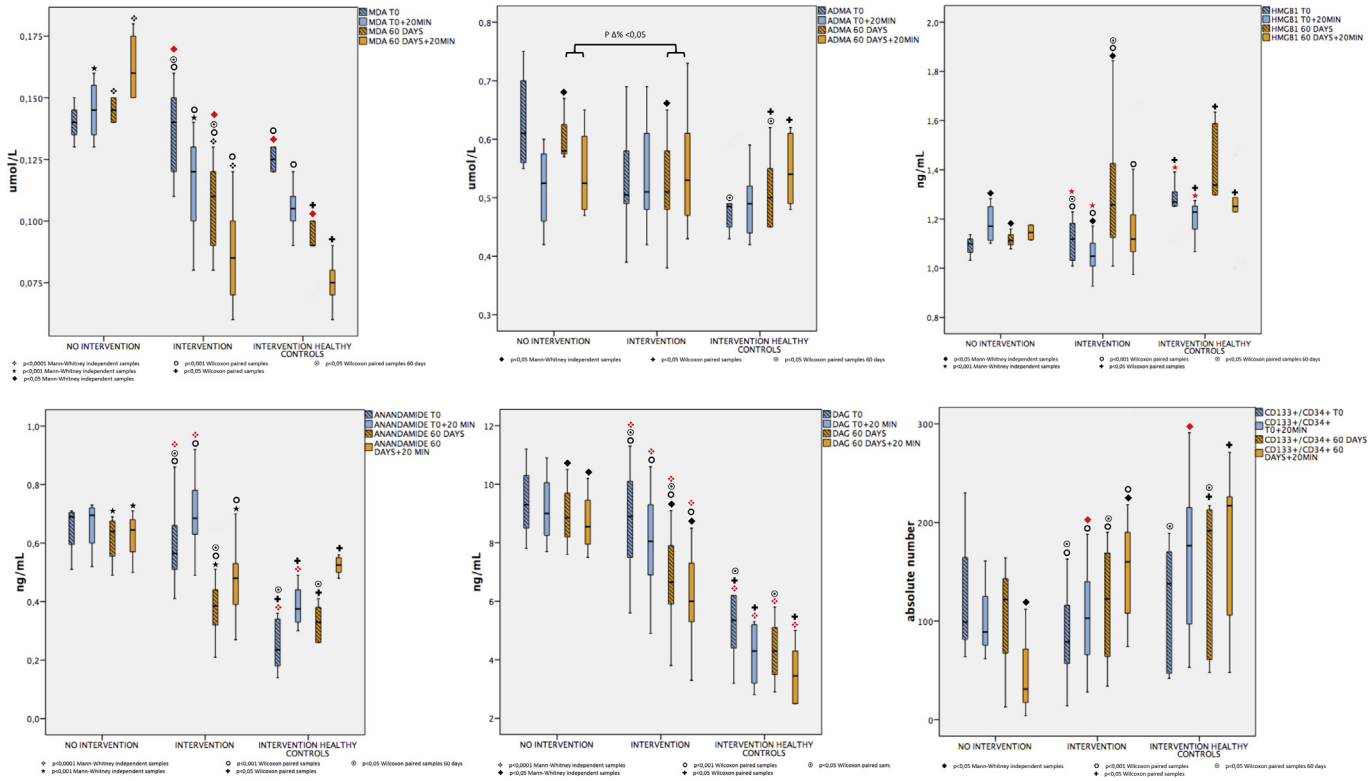

Fig. 5. MDA, ADMA, HMGB-1, anandamide, DAG and EPCs CD133+/CD34+ variation during the RR elicitation session at the time of the first observation (blue) and after 60 days (orange). Abbreviations as in the text.

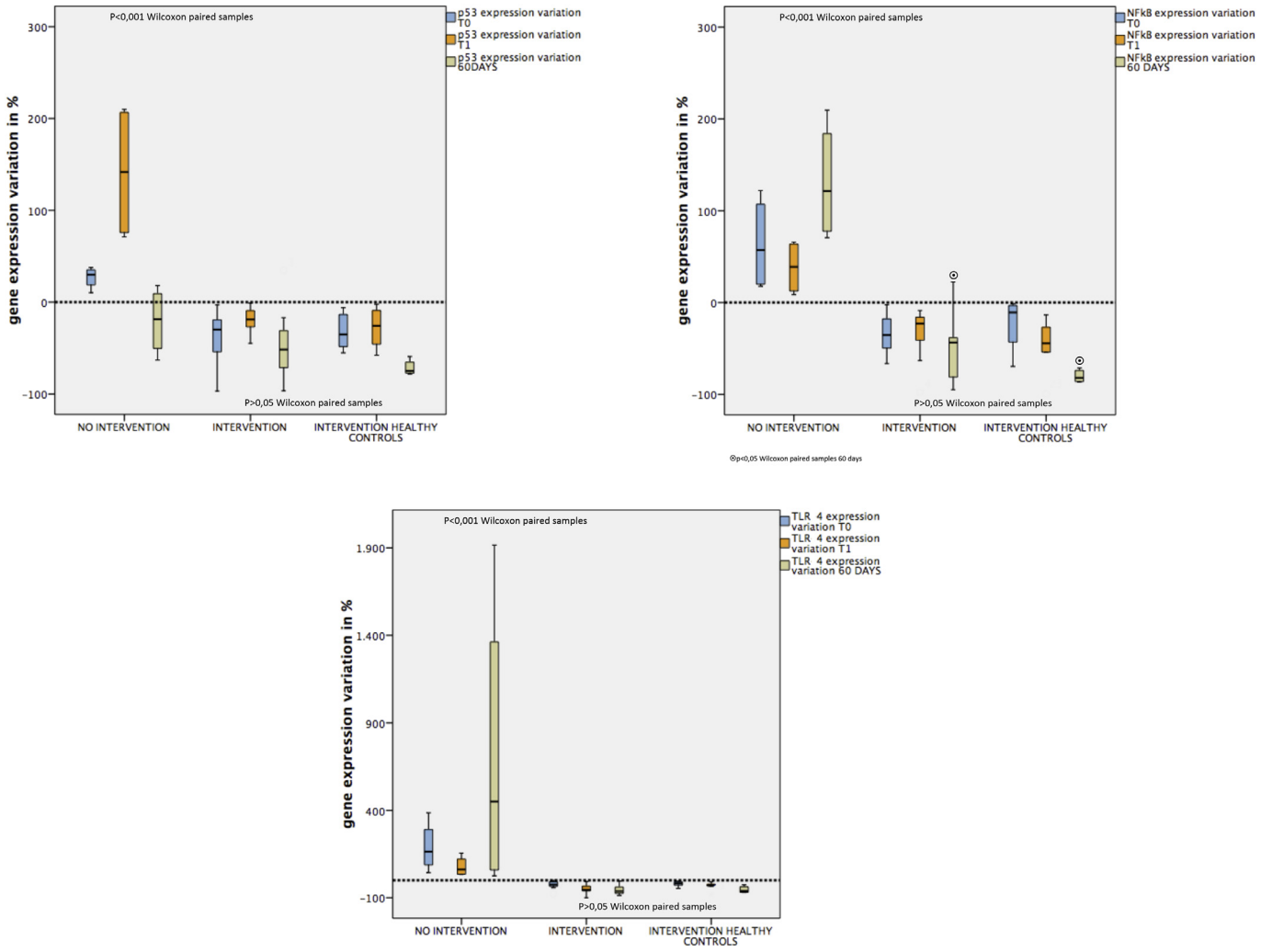

Fig. 6. Percentage change in the expression of p53, NFkB and TLR4 genes related to the Relaxing Response elicitation during the first observation (blue), the second observation (orange) and at 60 days (light brown). It's possible to notice an opposite trend in the control group of patients, probably related to the persistence of the activity of the brain areas of the Default Mode Network.

receptor.[31] Their signaling pathways are not fully elucidated but they can lead to changed expression of a variety of genes, including those involved in inflammatory responses and NO production.[31] In our study, patients have higher eCBs circulating levels at baseline than the healthy population, indicating the activation of a recovery mechanism triggered by myocardial infarction.[31] It is interesting to notice that the AEA increases during the RR while its metabolite 2-AG is reduced. Over time the values of AEA tend to shrink in the intervention group and tend to increase in the group of healthy individuals, almost certainly converging towards an equilibrium value related to the allostatic load[32] of the stress system. In control patients instead eCBs remain high and witness the persistence of a chronic activation of the alarm/stress system.

HMGB1 is a *damage-associated molecular patterns* (DAMP) molecule, able to contribute to both inflammation and tissue repair or regeneration.[33] Its activity varies depending on its redox state: when HMGB1 is oxidized, activates NFkB gene and is secreted in the blood and in the extracellular matrix regulating the immune homing in the site where the alarm occurred.[33] In reduced state it rules regenerative and reparative processes.[33] The stress signals that arrive at the cellular level can be physical (ex. infection) but may also be related to mental stress (sterile inflammation) and trigger a unique response of the cell called "universal cellular stress response" in which DAMPs molecules operate.[34] This study demonstrates that HMGB1 decreases switching off the mental stress through the practice of RR, witnessing a decrease of circulating danger signals in the body. After 60 days its basal levels have increased in the "intervention" groups. This behavior should be seen in relation to the decrease of oxidative stress and the attenuation of inflammation. It is likely that HMGB1 is increased but it is in a reduced state performing a riparative/regenerative action.

The whole cascade of messengers described converges on a cellular level by regulating the expression of our genome. This study considered three key genes that mediate the chronic low-grade inflammation process in turn involved in the pathogenesis of atherosclerosis and associated to mental stress.[1–3] While mental stress is able to increase the expression of NFkB,[35] the RR is capable of reducing the expression of p53, NFkB[8] and TLR4. NFkB and p53 are bound by a mutual inhibitory feedback. This makes it possible to read the change of p53 at 60 days in the control group of patients. In this group there is a decreased expression of p53 that must be put in relation to an increased expression of NFkB. Conversely, in the intervention groups there is a decreased expression of p53 with equally reduced NFkB, in line with the reduction of the circulating messengers of danger/stress.

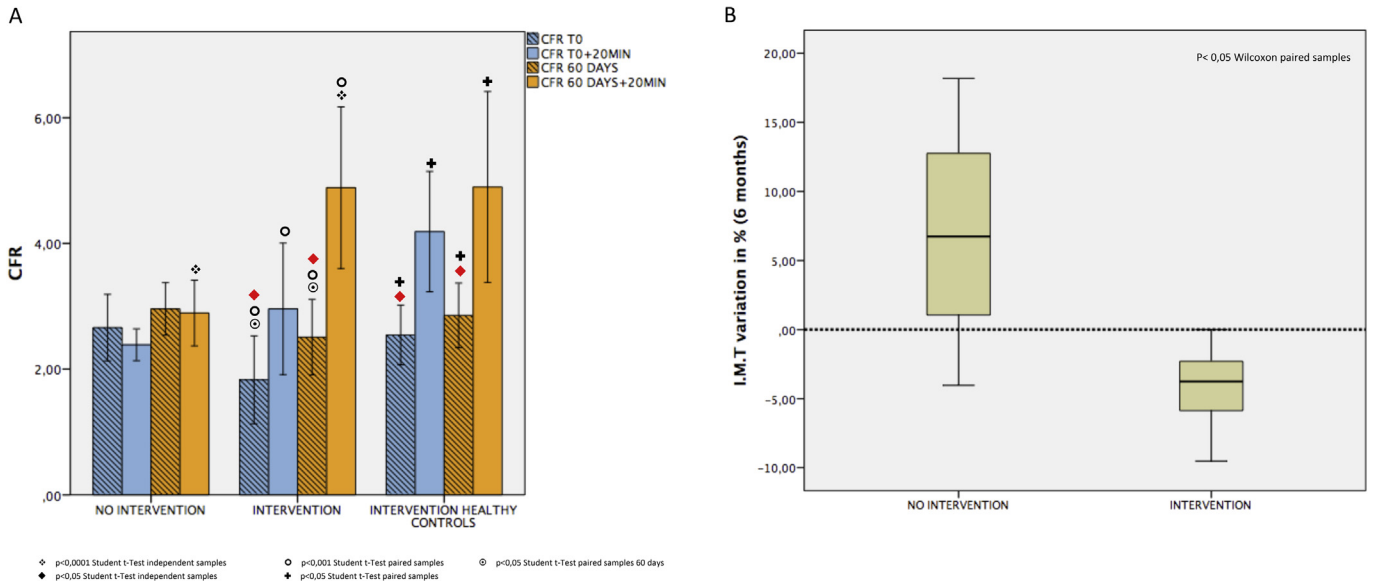

Fig. 7. A – CFR values in the three groups studied before and after the relaxation session at the first observation (blue) and the second observation after 60 days (orange). B – percentage change in IMT after six months of Relaxing Response practicing in comparison with the control group of patients.

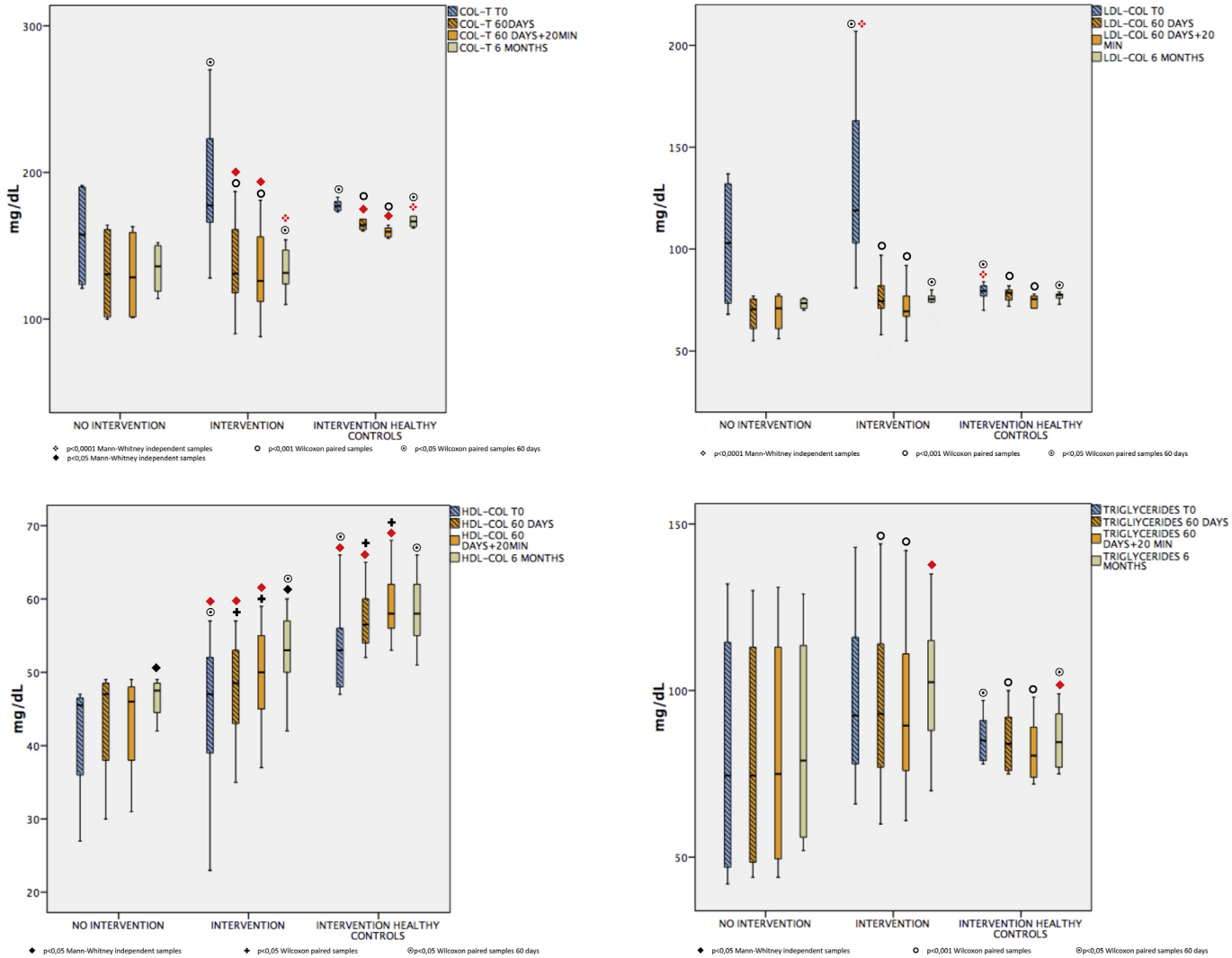

Fig. 8. Lipid profile variation between the first observation (blue), during the RR elicitation session after 60 days (orange) and after 6 months. Abbreviations as in the text.

EPCs CD34+/CD133+ increase with the RR, protecting patients from the possibility of a new coronary syndrome.[36] As demonstrated, their title varies in relation to the levels of norepinephrine[37] and, accordingly, an increase in inverse proportion to norepinephrine levels occurs. Their action maintains healthy vascular endothelium, especially from damage induced by oxidative stress.[38,39] They also prevent apoptosis of mature EPCs (that display the receptor KDR) trying to maintain a constant circulating level of KDR+ cells in order to reduce cardiovascular risk.[40–42] Thus, the maturation chain of EPCs seems to follow the trend of the physiological reaction to stress with levels of precursor cells that will likely differ depending on the alarm, compensation or exhaustion state of the organism.[1–3] The authors speculate that when their title starts to drop, their maintenance action is less on more mature KDR+ cells that in turn begin to shrink, increasing cardiovascular risk.

Even the lipid profile varies favorably with relaxation and, in this case, a mirror behavior to stress takes place. Indeed, stress is a contributor to chronic dyslipidemia and increases COL-T by 7 mg/dL and LDL-COL by 5 mg/dL within an hour of acute psychological stress.[43] According to our data we can say that the COL-T can undergo a variation of at least 13 mg/dL (+7 mg/dL during stress and −5 mg/dL during relaxation) in function of a psychic activity.

The observed changes in the circulating levels of NEI messengers occur below the presumed threshold of “normality”, especially at the time of the second observation. This suggests that clinically it may be more beneficial to rely on the subjective variation of the parameters analyzed over time rather than trying to establish a reference cut-off.[44,45] Indeed, when taking a blood sample, it is like capturing a still frame from a movie this is a dynamic process. Each molecule that circulates in our bodies continuously brings a message that has no particular limits or thresholds. Most likely, it is not the absolute amount of a mediator to determine the message, but the message itself to determine the need, at different times of the day, of life, or at different stages of stress response, more or less molecules to be transmitted.

Finally, the results related to oxidative stress, to the genetic expression and the regression of atherosclerosis indicate that there is no presence of a defect in the genome. A condition of cellular oxidative stress may lead to the wrong folding of proteins[46] with relative function deficits. Numerous genetic linkage studies do not take into account the effects of mental stress on oxidative stress and may have determined, for different pathologies, different gene-“diseased” protein associations that are entirely arbitrary because the problem may not necessarily reside in coding information but in the environment where the transition to the quaternary-functional protein structure happens. And this process is potentially reversible, as demonstrated in this study (confirming the data already published by Ornish et al.[47–49] and Castillo-Richmond et al.[50]). This would mean that the origin of the arteriosclerotic process may not necessarily be any inherent defect in the genome but a potentially reversible process whose possible onset and inheritance is based on epigenetics.[51]

The disease, in many cases, is the product of the individual way of thinking and living and not an external force or an internal fault. This process was also known to William Harvey, who wrote in 1628: “Every affection of the mind that is attended with either pain or pleasure, hope or fear, is the cause of an agitation whose influence extends to the heart”.[52]

5.3. Limitations

This study has some limitations. Mainly, the low number of patients with abnormal CFR limits statistical power. However, this was offset by the fact that each patient was studied twice and the second observation has reinforced the findings of the first. In addition, we looked at some key points of the integrated NEI network finding a coherent movement of the analyzed messengers. Moreover, the study was not randomized, but the practice of relaxation techniques requires a patient's voluntary decision that hinders a randomization process. We tried to overcome this aspect enrolling patients consecutively assigning them randomly to meditation or music appreciation after obtaining their consent to the study.

6. Conclusions

In summary, mental stress represents a key element in the pathogenesis of atherosclerosis that shows an initial regression after 6 months of regular practice of the anti-stress methods employed.

Our results suggest that the psychic activity and the orientation of our ongoing internal dialogue modulate the expression of our genome, and specifically of inflammatory genes. In particular, the RR achieved with meditation or music appreciation seems to improve the endothelial function in patients with CAD.

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Disclosures

None for all the authors. No sex-based or race/ethnicity-based differences were present.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jtcme.2017.04.011.

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